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Biocompatible, Biodegradable, and Enzymatic-Cleavable MRI Contrast Agents for Early Detection of Bone Metastatic Breast cancer

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14. ABSTRACT

This concept award proposes to design and test a novel peptide-based MRI contrast agent for early detection of bone metastasis from breast cancer. The proposed imaging agent is consist of bone targeting moiety of Asp8 and MRI imaging moiety of DOTA(Gd) with a cathepsin K cleavable peptide spacer. A solid phase peptide synthesis (SPPS) strategy was used to synthesize the peptide. Non-targeting and CTSK-insensitive controls were similarly prepared. The obtained imaging agent with a peptide sequence of Asp8CRPGGG has shown high binding affinity to hydroxyl apatite as well as biodegradability in the presence of cathepsin K. However, it also exhibited undesirable Gd chelation ability likely due to Asp8, resulting in its toxicity and limiting its use as a contrast agent for MRI applications.

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Peptide, MRI, Breast Cancer

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Introduction

Metastatic breast cancers in bone are very difficult to treat and result in significant morbidity and mortality. Current therapeutic options for breast cancer induced bone-metastases are usually palliative, and planning an individual therapeutic strategy as early as possible to delay skeletal complications is the key to manage patients with bone metastases. Hence, more specific imaging technologies to early detect and accurately diagnose whether and what cancerous bone metastasis is greatly needed in clinical practice. Currently skeletal scintigraphy represents the first line of diagnostic tools in the detection of osseous metastases. However, it suffers from poor sensitivity to lytic bone tumors, which is the case for breast cancer metastases. It also cannot provide structural details of lesions in bone. As a result, MRI is emerging as an alternative imaging modality for bone metastases diagnosis. Contrast enhanced MRI (CE-MRI) assisted with imaging probes can significantly improve early detection of tumor. However, MRI used for the diagnosis of bone metastases in the current clinical setting, has not yet involved the use of contrast agents. Thus, the development of appropriate bone-specific MRI probes for bone metastases is of importance. This proposal addresses this important public health need.

The central hypothesis of this project is that Gd complex in conjugation with a peptide which contains Asp_8 moiety and a cathepsin K (CTSK) substrate will achieve a novel bone-targeted, biodegradable and enzymatic-cleavable MRI contrast agent (Figure 1). Glycine terminal provides an amine group to attach the Gd chelator of DOTA. Asp_8 has a high affinity for bone mineral and has been used as bone-targeting moiety in molecular therapeutics.(*1-6*) The use of Asp_8 allows active accumulation of contrast agents in bone, thus leading to enhanced MR imaging in skeletal tissues. More importantly, by utilization of a CTSK-sensitive peptide linkage, Gd complex will be released following enzymatic cleavage. This not only reduces potential toxicity, but also leads to dynamic contrast enhancement at bone resorption sites to better detect CTSK activity. As CTSK is suggested as an indicator of bone metastasis from breast cancer,(*4, 7-14*) probing CTSK activity at bone turnover sites may suggest the pathogenesis of bone metastasis. In summary, the research proposes a new bone specific contrast agent which might be useful for early detection of bone metastatic breast cancer.

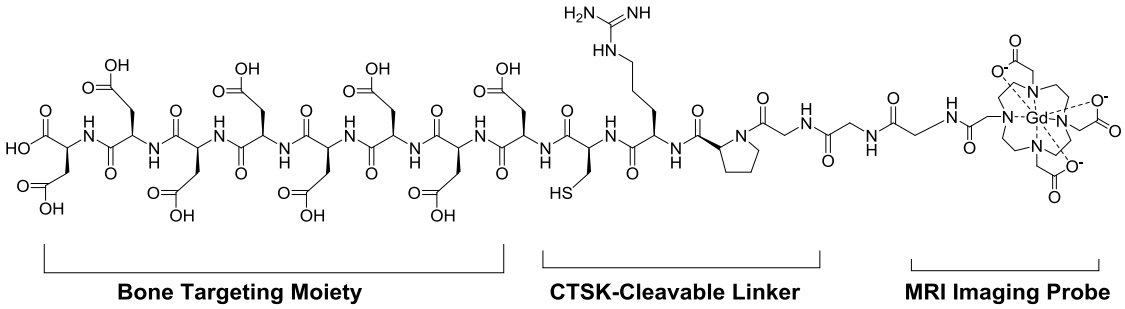


Figure 1: The concept of new bone-targeted, enzyme-degradable MRI imaging probe

Body

The first step for the project is to synthesize and purify peptide-based imaging agents. Proteolysis has been developed as a powerful tool for advanced drug delivery/imaging systems. Cathepsin K (CTSK), a protease primarily responsible for bone resorption, has been identified as a valuable therapeutic target for osteoclast-mediated osteolytic disease.(*10, 13, 14*) Hence, CTSK cleavable peptides have been explored in the development of molecular imaging probes and drug delivery systems in order to facilitate imaging agents/drugs release in osteolytic microenvironments.(*5, 7, 10, 11, 15, 16*) Oligo-peptides, e.g. RPGG (*7, 17*), NPGG (*5*) and HPGGPQ (*15*), have been demonstrated as CTSK-specific substrates. However, it is unclear which peptide sequence is more sensitive regarding to CTSK cleavage. To address this issue and identify best possible CTSK-cleavable linker for our project, we have initially synthesized CTSK-activatable imaging agents based on fluorescence resonance energy transfer (FRET) mechanism. We found that upon CTSK treatment, fluorescence intensity increased up to 8.2, 2.5, and 7.6- fold in the case of using RPGG, NPGG and HPGGPQ as substrate, respectively. This study confirmed literatures' results that all of above mentioned peptides are more or less sensitive to CTSK treatment (*5, 7, 15-19*), making all of them suitable as CTSK-sensitive peptides for drug delivery or molecular imaging applications. Originally we proposed to use NPGG as CTSK substrate. Both NPGG (*5*) and RPGG (*17*) were reported to be a CTSK-cleavable peptide sequence, based on our own practice, however, we made appropriate adjustment to switch NPGG to RPGG to obtain imaging agents. Accordingly, we have prepared 3 peptides (Figure 2) which were synthesized by solid phase peptide synthesis (SPPS) strategy, and purified by preparative HPLC. The chemical structures of peptides were shown in Figure 2. The molecular weights of these peptides were characterized and confirmed by mass spectroscopy (Figure 2).

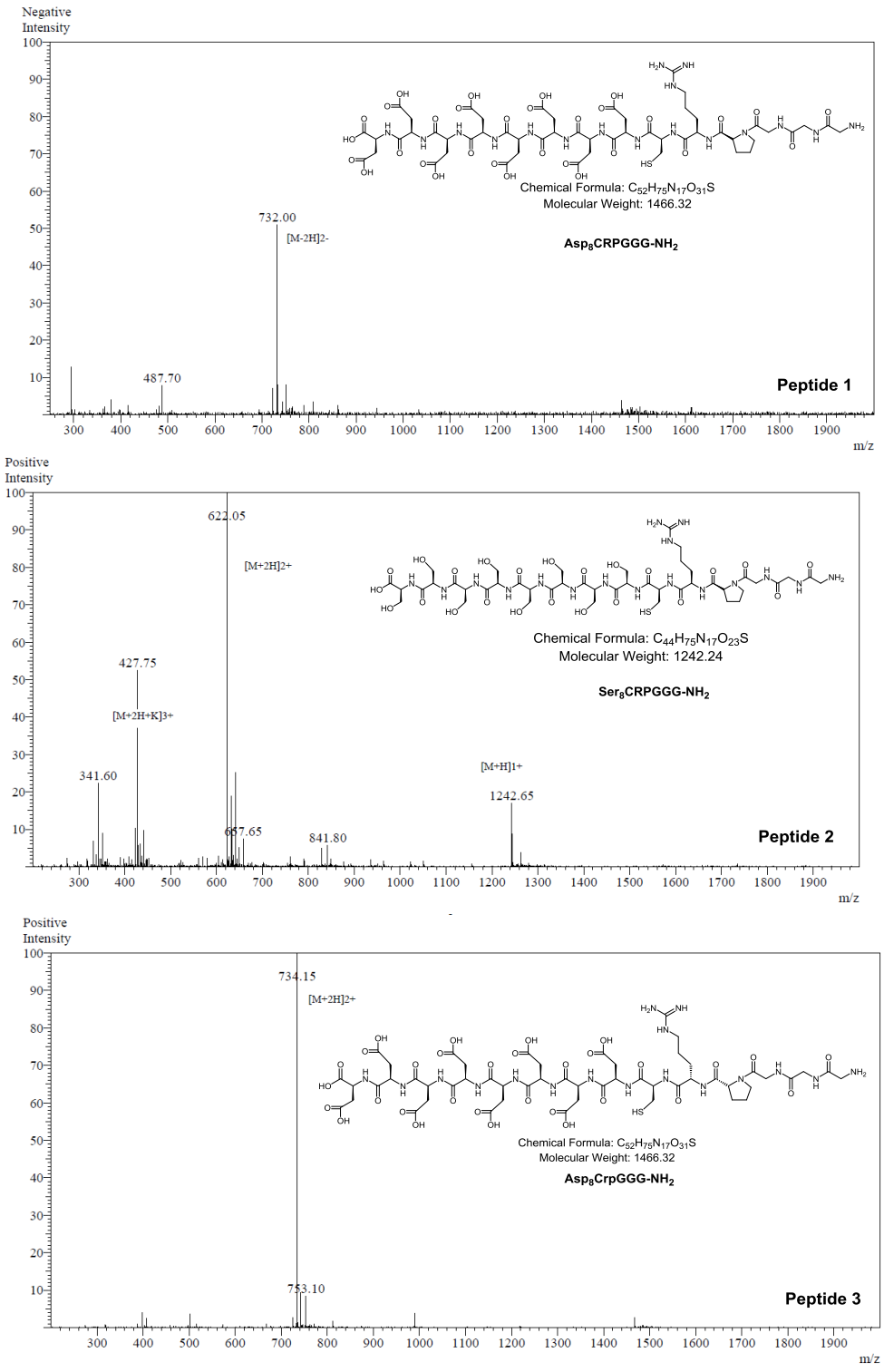


Figure 2: Mass spectra characterization of peptides which were synthesized based on SPPS strategy.

The most remarkable feature of skeletal tissue is its mineralized extracellular matrix. In the body, 99% of the calcium is located in bone as a component of mineral composition (apatite, $Ca_{10}(PO_4)_6$). To this end, a number of agents with high apatite binding affinity, including bisphosphonates (BPs) and oligopeptides (Asp_6, Asp_8 and Glu_8), have been identified to selectively bind to skeletal tissues in the development of water soluble bone-targeted drug delivery systems.(*20-25*) Based on these findings, we have proposed to use Asp_8 as bone-targeting moiety for imaging agents and tested the binding affinity of these imaging agents to hydroxyl apatite (HA). Mineral content (mainly apatite) accounts for nearly ~70% of the weight of fresh bone, so the binding affinity of nanotherapeutics to hydroxyapatite can mimic the binding affinity of nanotherapeutics to the bone.(*4*) To facilitate the evaluation of HA binding affinity of imaging agents, we conjugated FITC into peptides and then measured their HA binding affinity according to a method reported in the literature.(*1*) As shown in Figure 3, after 30-min incubation of imaging agents with HA, the percent of imaging agents bound to hydroxyl apatite were estimated at 3.9 ± 1.8, 66.2 ± 2.1, 15.5 ± 1.5, and 54.7 ± 2.7 for free FITC, Asp_8CRPGGG-FITC, Ser_8CRPGGG-FITC, and Asp_8CrpGGG-FITC, respectively. The data suggested that imaging agents with Asp_8 residue (bone targeting moiety) indeed possess high affinity to HA. In contrast, free FITC or the control peptide with Ser_8 residue only showed minimal and non-specific binding onto HA.

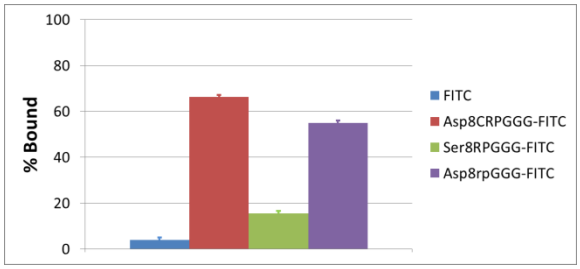
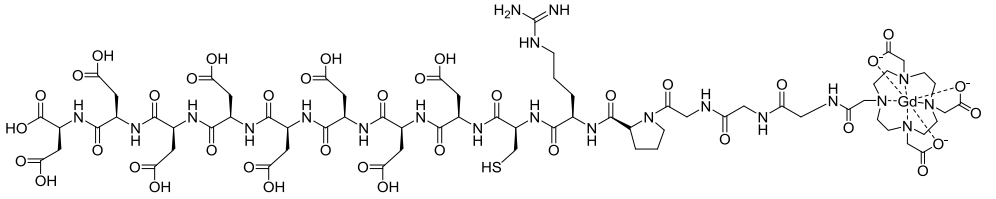


Figure 3: In vitro HA binding ability of imaging agents.

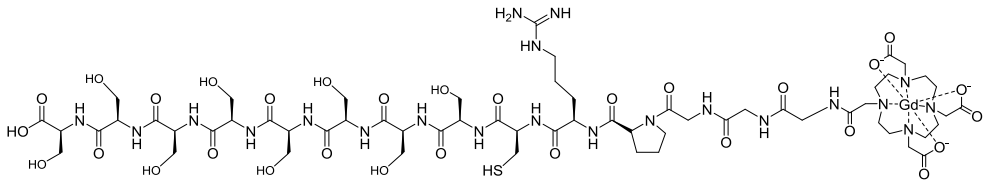
Next we performed experiments to investigate catepsin K-induced degradbility of imaging agents according to a similar method in the literature.(*5*) For this, FITC was conjugated to peptides as a model imaging moiety and its release from peptide backbone was monitored by HPLC. The imaging agents of Asp_8CRPGGG-FITC, Ser_8CRPGGG-FITC and Asp_8CrpGGG-FITC, which either contains CTSK-cleavable linker (RPGG) or CTSK-insensitive linker (rpGG), were incubated with cathepsin K (100 nM) at 37^{o}C in acetate buffer (0.1 M, pH 5.5). Cathepsin K was pre-incubated at 37^{o}C for 5 min to activate the enzyme in the active site, followed by addition of the imaging agents of Asp_8CRPGGG-FITC, Ser_8CRPGGG-FITC and Asp_8CrpGGG-FITC. HPLC analyses were performed on the Agilent 1200 series. HPLC apparatus equipped with a reverse-phase column (ZORBAX 300SB-C18, 5 µm, 4.6 × 150mm) and a Variable Wavelength Detector (VWD). The FITC moiety was detected at 490 nm. Cathepsin K catalyzed release of FITC fragments occured in the cases of RPGG-containing peptides, i.e. Asp_8CRPGGG-FITC and Ser_8CRPGGG-FITC, but not in the case of Asp_8CrpGGG-FITC. After 8-h post cathepsin-K treatment, it was calculated that approximately 56.5 ± 1.6, 62.8 ± 1.4, and

1.7 ± 0.5 % of FITC fragments were released from peptides of Asp_8CRPGGG-FITC, Ser_8CRPGGG-FITC and Asp_8CrpGGG-FITC, respectively. These results demonstrated that peptides with a sequence of RPGG are biodegradable in the presence of cathepsin K, which is consistent with the findings in literature.(*4, 7, 17*)

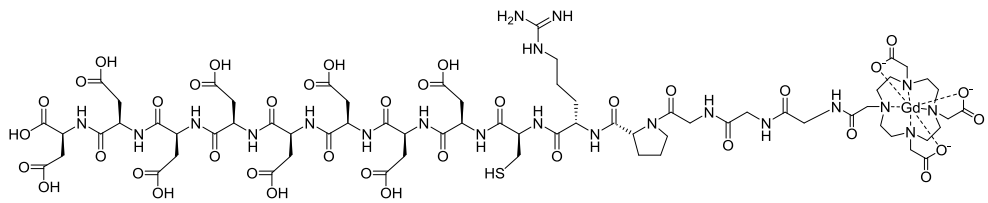
To obtain imaging agents for MRI studies, the above mentioned peptides were allowed to react with DOTA-NHS followed by complexing with $Gd(OAc)_3$ to afford proposed peptide-based MRI contrast agents (Figure 4). The mixtures were purified by dialyis (MWCO = 1000) against de-ionized water for 96 hrs. The final products were obtained after lyophilization. The gadolinium contents in the conjugates were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). The Gd contents of imaging agents were 1.33 ± 0.15, 0.51 ± 0.12 and 1.36 ± 0.16 mmol Gd/g, for Asp_8CRPGGG[DOTA-Gd], Ser_8CRPGGG[DOTA-Gd], and Asp_8CrpGGG[DOTA-Gd], respectively. The results were a bit to our suprise since the therotical maximum Gd loading capacity for the corresponding imaging agents are 0.50, 0.56 and 0.50 mmol Gd/g of compound. The data indicated that Gd conjugation degree for Asp_8CRPGGG[DOTA-Gd], Ser_8CRPGGG[DOTA-Gd], Asp_8CrpGGG[DOTA-Gd] were 267.1%, 91.1% and 273.1%, respectively, as compared to therotical Gd loading capacities which can be calculated based on chemical structures of imaging agents shown in Figure 4. The results suggested that peptide of Asp_8, but not Ser_8, maybe also involved in Gd cleation process. Therefore, a contruct of $Asp_8[Gd]_x$RPGGG[DOTA-Gd] or $Asp_8[Gd]_x$rpGGG[DOTA-Gd], which a certain degree of Gd was undersibely bound to Asp8 moiety, may be obtained.



Asp8CRPGGG-[DOTA-Gd]



Ser8CRPGGG-[DOTA-Gd]



Asp8CrpGGG-[DOTA-Gd]

Figure 4: Chemical structures of peptide-based MR imaging agents.

The *in vitro* cytotoxicity of imaging agents before and after Gd loading were evaluated in a breast cancer cell line of MDA-MB-231-Luc cells by a PrestoBlue™ (Invitrogen) cell proliferation assay. As shown in Figure 5, all imaging agents before Gd loading exhibited minimal cytotoxicity to cells at concentration up to 1000 μg/mL. However, after Gd was loaded into these imaging agents, mild cytotoxicities were observed at hight concentration (1000 μg/mL) in the case of Asp_8-containing imaging agents, in particularly for Asp_8CRPGGG-[DOTA-Gd] (Figure 5).

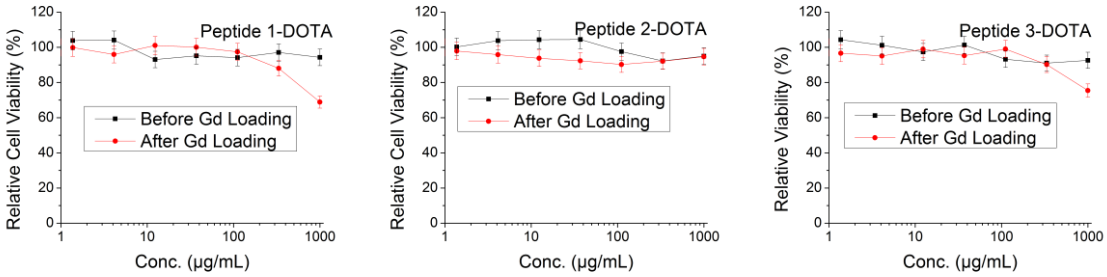


Figure 5: Cytotoxicity of peptide-based imaging agents in MDA-MB-231-Luc cells.

The above results, especially excessive and undesirable bound Gd onto Asp_8-containing peptides, have raised a safety concern for the use of these imaging agents in animals, since free gadolinium is very toxic in the body.(*26-29*) Therefore, we have performed a tolerated dose study to investigate if these imaging agents would cause acute toxicity to animals at a clinical meaningful MRI testing dose (0.1 mmol Gd/Kg). Imaging agents of Asp_8CRPGGG[DOTA-Gd] and Ser_8CRPGGG[DOTA-Gd] in 100 μL of PBS were injected into mice through tail vein at a dose of 0.1 mmol Gd/kg (3 mice per group). Equivalent amount of Asp_8CRPGGG-DOTA without Gd loading was used as a control (3 mice). Unfortunately, all the mice that were administrated with Asp_8CRPGGG[DOTA-Gd] died within 2 to 24 hours after injection. In contrast, no obvious toxicity was observed for those mice received either Ser_8CRPGGG[DOTA-Gd] or Gd-free peptide of Asp_8CRPGGG-DOTA. It appears that acute toxicity of Asp_8CRPGGG[DOTA-Gd] likely resulted from excessive bound Gd ions onto Asp_8CRPGGG-DOTA. We have originally proposed to use Asp_8 as bone targeting ligand (Figure 1). Unexpectedly, Asp_8 not only possesses HA binding affinity (Figure 3), but also appears to be able to chelate with Gd ions, leading to a construct of $Asp_8[Gd]_x$RPGGG[DOTA-Gd] (Figure 6). However, Asp_8 is unlikely to be a Gd chelator as strong as DOTA. Such imaging agents may release free Gd ions *in vivo* in the presence of competing ions such as Ca^{2+}, Fe^{2+}, Fe^{3+}, and others (Figure 6). Such a drawback raises a significant safety issue for the use of Asp_8-containing MR imaging agents in animals. Therefore, although $Asp_8[Gd]_x$RPGGG[DOTA-Gd] indeed possesses high bone-targeting ability and Cathepsin K-induced degradability, it is not suitable as a safe agent for MRI applications.

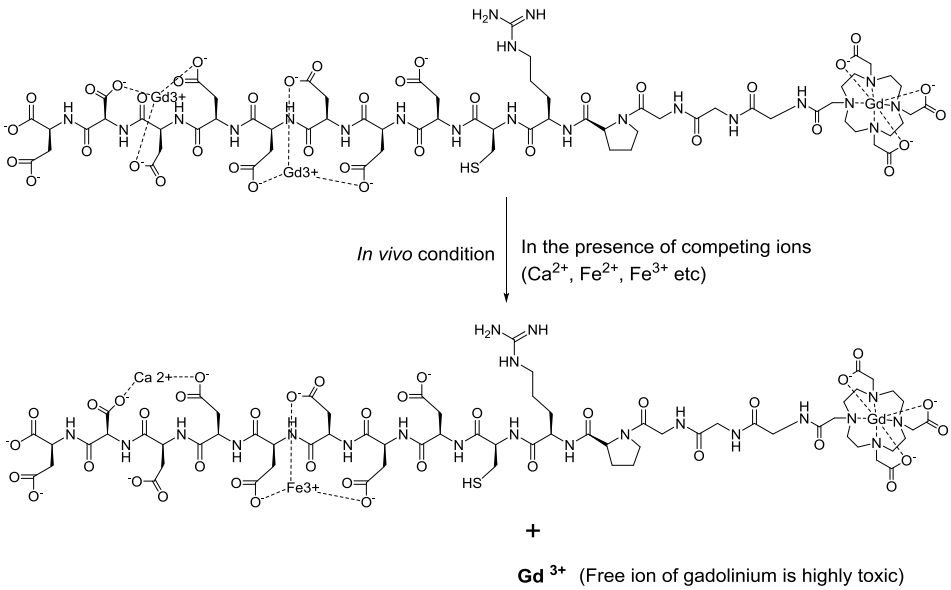


Figure 6: Possible mechanism of action of $Asp_8[Gd]_xRPGGG[DOTA\text{-}Gd]$ induced toxicity

Key Research Accomplishments

- Peptide-based imaging agents were successfully obtained.
- Asp_8-containing peptides were confirmed to have high HA binding affinity.
- HPLC studies revealed that RPGG is a valid substrate for cathepsin K.
- $Asp_8[Gd]_xRPGGG[DOTA\text{-}Gd]$, although it possesses high bone-targeting ability and Cathepsin K-induced degradability, may not be a good candidate for MRI applications because of a safety issue.

Reportable Outcomes

None

Conclusion

This concept award project proposes to design and test a novel peptide-based MRI contrast agent. We have successfully obtained peptides by using a solid phase peptide synthesis (SPPS) strategy. Non-targeting and CTSK-insensitive controls were similarly prepared. The obtained contrast agents were characterized in terms of bone specificity, enzymatic degradability and biocompatibility. The proposed MRI imaging agent, i.e. Asp_8[Gd]xRPGGG[DOTA-Gd], although it possesses high bone-targeting ability and Cathepsin K-induced degradability, may not be a good candidate for MRI applications because of a safety issue.

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